EXHIBIT A82

Human Reproductive Biology

THIRD EDITION

Richard E. Jones
Professor of Biology Emeritus
University of Colorado
Boulder, Colorado

Kristin H. Lopez
Department of Integrative Physiology
University of Colorado
Boulder, Colorado

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CHAPTER NINE

Gamete Transport and Fertilization

Introduction

The process of *fertilization*, or *conception*, involves fusion of the nucleus of a male gamete (sperm) and a female gamete (ovum) to form a new individual. Because each gamete is haploid (N), fertilization restores the normal diploid (2N) chromosomal complement. Fertilization, however, is more than the simple fusion of gametes in that it is preceded by and requires a series of precisely timed events. Once sperm are deposited in the female reproductive tract, they travel a relatively long distance and overcome several obstacles before reaching the ovum. Similarly, the ovum travels through a portion of the female reproductive tract before it is fertilized. Not only do the gametes move to the appropriate regions of the female tract, but they undergo important physical and biochemical maturations that are a prerequisite for fertilization. Abnormalities in these maturational or transport processes, as well as in fertilization itself, can lead to infertility, spontaneous abortion (miscarriage), or birth defects.

Semen Release

After leaving the epididymides, sperm enter the vasa deferentia, which are long paired ducts serving as sperm storage and transport organs (see Chapter 4). Secretions of the male sex accessory glands (*seminal plasma*) mix with the sperm during ejaculation to form *semen* or *seminal fluid*. It has been theorized that the entire reserve of sperm in the epididymides and vasa deferentia would be depleted if an adult male had 2.4 ejaculations per day for 10 consecutive days. However, this normally does not occur, even with such Herculean ejaculation frequency because new sperm are produced continuously by the testes—about 200 million per day! Thus, frequent ejaculation is not an effective method of contraception.

Semen is released in three stages. Before male orgasm, a small amount of semen comes from the bulbourethral glands. In the second stage, the majority of semen is released; most of the seminal plasma of this stage comes from the seminal vesicles and prostate gland. In the third stage, another small amount of fluid produced by the seminal vesicles is exuded. Most of the sperm are expelled in the second stage, but some sperm are present in the semen of the first and third stages. Because sperm are present in the first stage, pregnancy can occur without male orgasm, which is one reason why *coitus interruptus* (withdrawal of the penis before ejaculation) is not an efficient method of birth control (see Chapter 14).

Contents of Seminal Plasma

Seminal plasma contains several substances, but the precise function of many of these components is not known. We do know, however, that some of them have roles in the maintenance, maturation, and transport of sperm. Water is present, which serves as a liquid vehicle for the sperm and seminal plasma constituents. Mucus from the bulbourethral glands serves as a lubricant for the passage of semen through the male reproductive tract. The prostate gland and the bulbourethral glands both secrete buffers, which neutralize the acidity in the male urethra and in the vagina. Some nutrients for sperm are present in the seminal plasma deposited in the vagina, the major ones being the sugar fructose and citric acid (from the seminal vesicles). *Carnitine*, concentrated from the blood by the epididymis, is also found in the seminal plasma. This chemical is involved in the metabolism of fatty acids, with the metabolites being used as another nutrient source for the sperm. Another constituent of seminal plasma secreted by the epididymis is *glycerylphosphocholine*. The enzyme *diesterase* in the uterus hydrolyzes (breaks down) this molecule, and the products of this digestion are used by the sperm as nutrients. Other enzymes secreted by the prostate gland and seminal vesicles are involved in the clotting and subsequent liquefaction of semen in the vagina. Human seminal plasma contains extremely high amounts of zinc (which may have antibacterial activity), and men with low zinc content tend to have a higher incidence of infertility. Finally, some kinds of prostaglandins are secreted into the seminal plasma, mostly by the seminal vesicles. Prostaglandins in seminal plasma may be involved in sperm transport. Finally, seminal plasma contains ATP, and men with low semen ATP levels tend to have lower fertility. Table 9-1 summarizes the sources of the major components of seminal plasma.

Table 9-1 Some Characteristics of Human Semen

General properties
- Creamy texture: gray to yellow color
- Average volume: 2.5–3.5 ml after 3 days of abstinence (range, 2–6 ml)
- Fertility index (minimum qualifications for male fertility):
  1. At least 20 million sperm/ml
  2. At least 40% sperm must show vigorous swimming
  3. At least 60% sperm must have normal shape and size
- pH: 7.35–7.50 (slightly basic)

Sources and major components of seminal plasma

Epididymis (a slight amount)	Seminal vesicles (about two-thirds of total volume)	Prostate gland (about one-third of total volume)	Bulbourethral glands (a few drops)
Water	Water	Water	Water
Carnitine (a nutrient)	Fructose (a nutrient)	Bicarbonate buffers (neutralize vaginal pH)	Buffers (neutralize vaginal pH)
Glycerylphosphocholine (a nutrient)	Fibrinogen (clots semen)	Fibrinogenase (clots semen)	
	Ascorbic acid (a nutrient)	Fibrinolytic enzyme (liquifies semen)	Mucus (lubrication)
	Most of the prostaglandins (contract the vas deferens)	Citric acid (a nutrient)	
		A little prostaglandin	

Sperm Number and Structure

The number of sperm in a single ejaculate ranges from 40 million to 500 million (the average is about 182 million sperm). A male produces about 1 billion sperm (Fig. 9-1) for every ovum ovulated by a woman. Many ejaculated sperm (about 30%), however, are structurally or biochemically abnormal and are either dead or incapable of fertilizing; these are reabsorbed by the female reproductive tract or are lost through the vagina. For a male to be minimally fertile, his sperm count should be at least 20 million sperm/ml of semen; 40% of these sperm must swim and 60% should be of normal shape and size (Table 9-1).

Some evidence shows that human sperm count has declined over recent decades (see Chapter 4). One study suggests that the sperm count in healthy men has dropped 1% per year in the past 50 years. However, other studies contradict this idea, and whether there has been a worldwide decline in male fertility remains controversial. It is clear, however, that geographical differences in average sperm count exist. Differences in sperm production of men living in disparate regions of the world may reflect genetic, cultural, or environmental differences.

A healthy human sperm is 40 to 250 μm long and is composed of the following structures (Fig. 9-2): neck, midpiece, and tail. The *sperm head* contains an elongated haploid nucleus surrounded by a nuclear membrane. External to the nucleus is a membrane-bound vesicle called the *acrosome*. It fits closely over the tip of the sperm head like a cap, and the *inner acrosomal membrane* lies external to the nuclear membrane while the *outer acrosomal membrane* is just inside the sperm cell *plasma membrane*. The acrosome is filled with enzymes important in the penetration of the ovum. The short sperm neck is followed by the sperm midpiece, which contains mitochondria that generate energy for tail movement. The midpiece and sperm tail represent a flagellum, with the "9 + 2" arrangement of microtubules. This provides the propulsive force, allowing locomotion of the sperm cell as it moves toward the egg and during egg penetration. A human sperm cell is 60–70 μm in length.

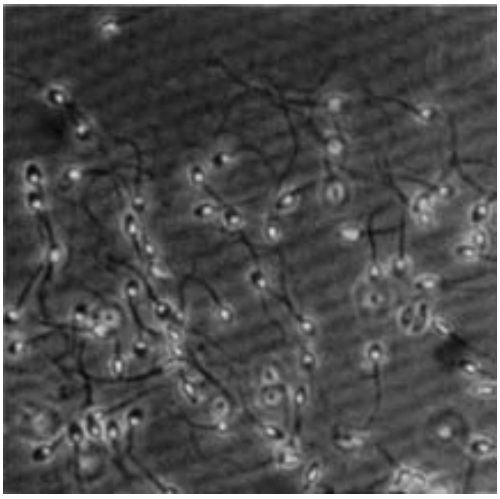


Figure 9-1 Photomicrograph of human sperm swimming in seminal fluid. The sperm heads shine because of a fluorescent dye.

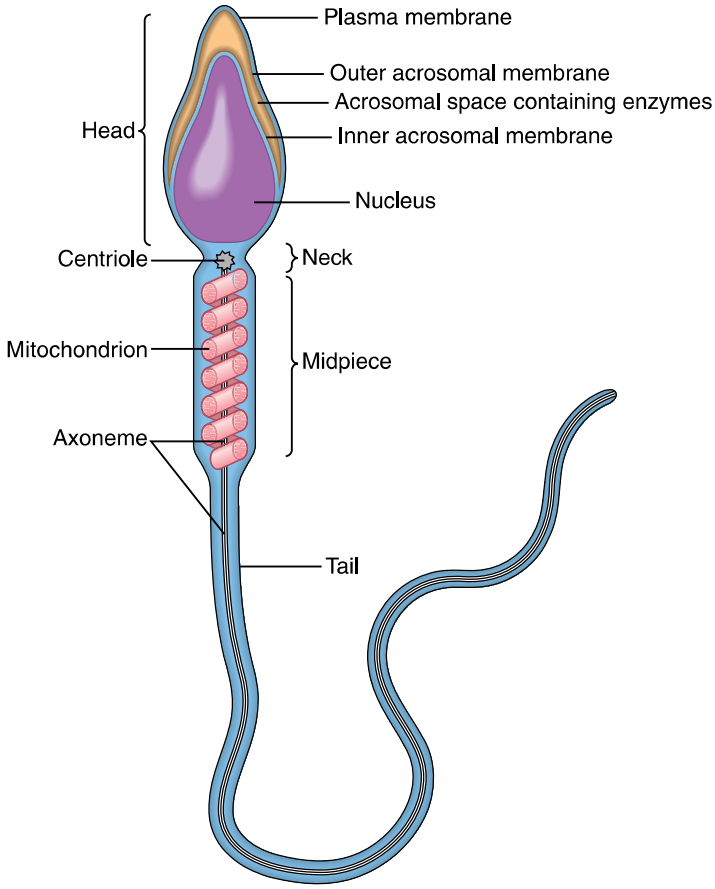


Figure 9-2 Sperm structure.

Sperm Transport and Maturation in the Female Reproductive Tract

Let us now follow the sperm cells on their journey through the female reproductive tract to the point of fertilization in the oviduct, a distance of about 15 cm (6 in.). The sperm are first deposited in the vagina; they then pass up this cavity and through the cervix into the uterus, up the uterus, through the junction between the uterus and oviduct (uterotubal junction), and up the isthmus of the oviduct to the usual area of fertilization in the oviduct: the ampullary–isthmic junction. Many of the millions of deposited sperm are lost during this journey, and only about 100 to 1000 reach the oviduct, with 20 to 200 reaching the egg itself. In addition, the sperm must undergo maturational processes during their journey, which give them the capacity to move and to fertilize an ovum.

Vaginal Sperm

About 1 min after deposition in the vagina, the semen becomes thicker and less liquid. This *semen coagulation* is brought about by the enzyme *fibrinogenase*

in the seminal plasma, which converts the protein *fibrinogen* to *fibrin*, another protein. The major function of this coagulation may be to prevent sperm loss from the vagina. After about 20 min, however, the semen again liquefies. This *semen liquefaction*, which is caused by a *fibrinolytic enzyme* in the seminal plasma, stimulates some sperm to swim more rapidly and to reach the cervix. Even though semen liquefaction has not yet occurred, some sperm make it into the cervix and even into the uterus within a few minutes of deposition in the vagina.

The environment in the vagina is usually acidic (about pH 4.2), and this level of acidity inhibits sperm motility. The presence of semen in the vagina, however, increases the vaginal pH to a basic 7.2, which in turn increases sperm motility.

During coitus, female orgasm is accompanied by muscular contractions of the vaginal walls (see Chapter 8), and these contractions create a pressure in the vagina that is higher than that in the uterus. Sperm movement through the cervix may be aided by this pressure differential. Sperm, however, can move up the female tract without female orgasm.

Cervical Sperm

The cervical canal is lined by a complicated series of narrow folds and crypts and is blocked by a sticky mass of cervical mucus and tiny cervical fibers (see Chapter 3). In most stages of the menstrual cycle, the mucus is thick and fibers within it are densely packed. Shortly before ovulation, however, the rise in circulating estrogen levels causes the mucus to become more liquid and the gaps between the cervical fibers to widen. These gaps orient so that channels are formed. When the sperm enter the mucus, they line up in these channels almost in single file and pass through the cervix at a speed of about 1.2–3.0 mm per minute.

The cervical fibers may serve as a network upon which the sperm tails exert force, beating with a spiral motion and thus propelling the sperm upward. Also, these fibers may be of such dimension and length that they vibrate in rhythm with the tail beat frequency of normal sperm; this may allow normal sperm to move through the cervix, whereas sperm with abnormal or absent tail beats are detained. These latter sperm then die and are reabsorbed or lost from the body. Other sperm enter *cervical crypts* (deep recesses in the cervical wall), where they die or are lost, or they may remain alive as a reservoir of sperm that later may enter the uterus. Fewer than 1 million of the original 182 million sperm make it through the cervix.

Uterine Sperm

Upon leaving the cervix, the sperm travel up the uterus to the uterotubal junction. The uterine fluid is watery but sparse in humans, and the sperm essentially "climb" up the uterine lumen by beating their tails. The swimming rate of sperm (about 3 mm/min), however, cannot account for their traveling a distance of about 15 cm in the 30 min after ejaculation. Also, dead sperm reach the oviduct at about the same time as do live sperm. Thus, sperm tail beating probably is not important during sperm transport through the uterus so it

must be the muscle contraction and movement of cilia in the female reproductive tract that facilitate sperm transport.

Mechanical stimulation of the cervix by the penis during coitus causes release of the hormone oxytocin from a woman's posterior pituitary gland. This hormone quickly travels via the blood to the uterus and increases the force of rhythmic uterine muscle contractions. These contractions act as waves to help the sperm move to the uterotubal junction. Prostaglandins in the seminal fluid may also cause uterine muscles to contract, but this is unlikely as very little if any seminal fluid enters the uterus through the cervix. The main function of the prostaglandins in seminal fluid is probably to contract the muscles of the vasa deferentia, thus aiding sperm passage during ejaculation.

The presence of sperm in the uterus initiates a massive invasion of white blood cells (*leukocytes*) into the uterine lumen. These cells then begin to engulf the dead or dying sperm that have not yet moved up to the uterotubal junction. No more than a few thousand sperm reach this junction.

The uterotubal junction is a muscular, tightly constricted region separating the uterus from the oviduct (see Chapter 2). Sperm enter the narrow opening of this junction and move through it at a relatively slow rate. Thus, the uterotubal junction allows the gradual entrance of sperm into the isthmus of the oviduct. About half of the sperm enter the wrong oviduct, and only a few hundred make it to the general proximity of the waiting egg.

Transport of the Sperm and Ovum in the Oviduct

Sperm tail beating is reduced, and the sperm "wait" in the isthmus for ovulation to occur. Other sperm previously residing in cervical crypts are also released around the time of ovulation. After ovulation, several sperm move up to the ovum, and fertilization by a single sperm usually occurs at the point where the isthmus joins the wider oviductal ampulla (ampullary–isthmic junction). Other sperm swim up the ampulla, through the infundibulum, and are lost in the body cavity.

Once ovulation has occurred, the infundibulum (funnel-shaped free end) of the oviduct moves to the ovary and envelops the ovulated ovum along with fluid derived from the ovulated follicle. Movement of the infundibulum is accomplished by the contraction of muscles in the membrane supporting the oviduct. Cilia are present in the wall of the fimbria (the edge of the infundibulum) and these beat toward the uterus. Thus, when the infundibulum envelops the ovary, the beating of the cilia moves the ovum into the ampulla of the oviduct. Cilia in the ampulla and isthmus of the oviduct also beat in a uterine direction, which sets up a flow of fluid toward the uterus.

The muscles of the oviduct also exhibit waves of muscular contraction after ovulation. These waves travel in the direction of the uterus and, along with the cilia, help the ovum move down the oviduct. Both ciliary beating and muscular contraction in the oviduct are influenced by ovarian sex hormones. Estrogens increase cilia number, and progesterone increases ciliary beating and egg transport.

A factor involved in the opposite movement of egg and sperm may be the direction of ciliary beating in the oviduct. Oviductal cilia exist in deep recesses in which cilia beat toward the ovary and on ridges where these cilia beat

toward the uterus. Sperm may travel in these recesses, whereas the ovum may be propelled along the ridges. The presence of considerable amounts of mucus in the oviducts for 3 to 4 days after ovulation may serve as a medium for sperm transport. This mucus is gone when the fertilized ovum (embryo) travels down the oviduct to the uterus, as discussed in Chapter 10.

Sperm Capacitation and Activation

Freshly ejaculated human sperm are not capable of fertilization. A period in the female reproductive tract is necessary before sperm can fertilize an oocyte. Thus, during their journey, sperm gain the ability to fertilize an egg (a process called *sperm capacitation*). *Calmodulin*, a protein in seminal plasma, may also play a role in sperm capacitation. This protein (or another epididymidal secretion) may give the sperm the ability to be capacitated later on when they are in the uterus.

In general, the present scientific opinion is that capacitation involves removal or modification of molecules (glycoproteins) associated with the sperm head that stabilize the sperm plasma membrane. These molecules suppress the ability of sperm to fertilize. Alteration or removal of these inhibitory molecules allows the sperm to respond to signals that trigger the acrosome reaction, an important step in the fertilization process. Capacitation also increases the vigor or tail movements of the sperm (*hyperactivation*), propelling it toward the egg more effectively.

What substances in the female reproductive tract render the sperm capable of fertilization? One possibility is that molecules in follicular fluid escaping from the ovulating follicle play a role in sperm capacitation. Follicular fluid contributes only a small part of the oviductal fluid. Studies of mammals have demonstrated that two components of follicular fluid, progesterone and the protein albumin, facilitate the acrosome reaction. Calcium in follicular fluid increases the vigor of sperm tail beating. It is not clear if these substances are present in humans, although follicular fluid does activate human sperm. If operative in humans, they may have their effect when the sperm penetrates the cumulus oophorus (see later), which surrounds the ovum and is bathed in follicular fluid. A recent discovery is that follicular fluid, or the egg itself, produces a chemical that attracts human sperm (see HIGHLIGHT box 9-1). Another study suggests that mammalian sperm move toward the egg along a thermal gradient. The site of fertilization is slightly warmer than more proximal portions of the oviduct, and mature sperm have a preference for moving toward warmer fluid (*thermotaxis*). Sperm may be guided by temperature during most of their journey through the fallopian tube and then respond to chemical cues as they near the egg. In the future, we may expand our concept of sperm capacitation to include acquisition of the ability to detect chemical and/or thermal cues.

When Can Fertilization Occur?

Most references state that sperm live about 72 h and that an egg is fertilizable for 24 to 48 h. Thus, the fertile time in a menstrual cycle would be about 4 to 5 days,

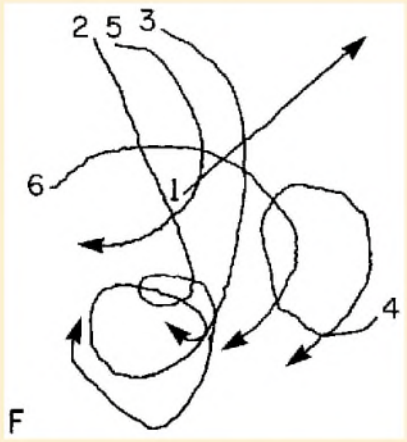
Chapter 9, Box 1: Does the Human Egg Court Sperm?

Out of the millions of sperm deposited in the vagina during ejaculation, only 20 to 200 will reach close proximity to the egg in the oviduct, and yet the competition among sperm to be the one that fertilizes the ovulated egg is not finished. Part of the future of a sperm (to become part of a new embryo or to die as a haploid failure) could depend on its response to "courtship" by the egg and/or its surrounding fluid.

The sperm of algae, mosses, ferns, and some invertebrate animals are attracted to the egg chemically, but until recently only one case of such attraction has been described in a vertebrate animal. The egg of the herring (a teleost fish) is covered by a zona pellucida-like coat (the "chorion"), and the chorion cannot be penetrated by sperm swimming in the surrounding water unless it locates a small opening in it. This opening, the micropyle, secretes a chemical that activates and attracts sperm to it. However, until recently, there had been no evidence that the human egg attracts sperm. The pervading theory had been that the human sperm present in the vicinity of the egg bump into it by chance.

A new finding, however, suggests that the human egg produces a chemical that attracts sperm and influences their swimming motion. If follicular fluid from a large grafian follicle is placed at one end of a chamber, sperm will accumulate at that end, whereas they will not respond to a control fluid. The quantities of estradiol or progesterone in the fluid do not influence this response, but only some and not all follicles have fluid that works. A good correlation also exists between the fertilizability of an egg and the ability of its surrounding fluid to attract sperm. Control fluid previously containing an egg also attracts sperm, so it appears that this signal comes from the egg, not the surrounding follicular cells.

When sperm are exposed to the egg signal, they swim in a circle instead of in a straight line, which would increase their chances of contacting the egg. Interestingly, not all sperm are attracted to the egg; some could care less and some even swim away from the egg! A human sperm has about 20 chemical receptor molecules on its head, and maybe some sperm have not formed the receptor(s) used in this chemical orientation to the egg or perhaps they are abnormal in other ways. Nevertheless, they will not be the chosen one! Many questions still remain. What is the chemical that attracts sperm? Why do some eggs produce the chemical and some not? Why do some sperm respond whereas others do not? Does the chemical cause more sperm to move up the oviduct leading to the egg instead of the "empty" oviduct? Do X and Y sperm behave differently in response to this chemical? Could an inhibitor of this chemical be used as a new contraceptive agent? Only time will tell.



Six human sperm were placed in a fluid-filled chamber. Their starting position is represented by the numbers 1 through 6. Then, either follicular fluid from a human Graafian follicle or fluid exposed to a human egg was injected at the lower left-hand corner (F). Arrowed lines then indicate the path swum by each sperm. Note that sperm 2 through 6 turned and headed toward F. Sperm number 1, however, was not interested. (Adapted from Ralt *et al.* (1991).)

with ovulation occurring at about the middle of this time period. A recent study, however, has cast suspicion on this theory. This study found that conception can only occur in a 6-day period, i.e., during the 5 days before ovulation or on the day of ovulation. Therefore, some sperm live for 6 days and the egg lasts 12 to 24 h (or the change in cervical mucus after ovulation halts sperm transport).

The Process of Fertilization

Once a sperm and ovum are in the region of the ampullary–isthmic junction of the oviduct (Fig. 9-3), fertilization occurs. In the fertilization process, a sperm first penetrates between the cells constituting the cumulus oophorus and then through the zona pellucida and into the perivitelline space. The sperm then enters the oocyte through its cell membrane (the *vitelline membrane*). The following is a discussion of what happens during each of these processes, and Figs. 9-4 and 9-5 depict these processes. The entire process of fertilization takes about 24 h.

Sperm Passage through the Cumulus Oophorus

The ovulated ovum is surrounded by the cumulus oophorus, which is a sphere of loosely packed follicle cells (Fig. 9-4). Appropriately, cumulus oophorus means "egg-bearing little cloud." As a sperm enters the cumulus oophorus, the enzyme *hyaluronidase* on the sperm head dissolves hyaluronic acid, a major component of the cementing material found between the cells of the cumulus oophorus as well as between other cells in the body. Enzymatic dissolution of hyaluronic acid allows the swimming sperm to penetrate the cumulus oophorus and to reach the zona pellucida.

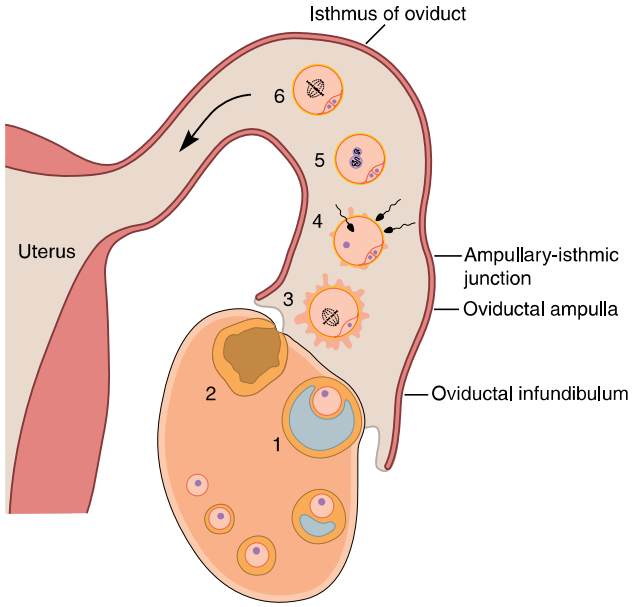


Figure 9-3 Diagram of the human ovary, oviduct, and part of the uterus showing fertilization: (1) Follicle in ovary is ready to ovulate; (2) new corpus luteum; (3) ovulated ovum is arrested in second meiotic division (note the first polar body); (4) formation of second polar body after fertilization; (5) fusion of egg and sperm pronuclei; and (6) beginning of first mitotic division of zygote.

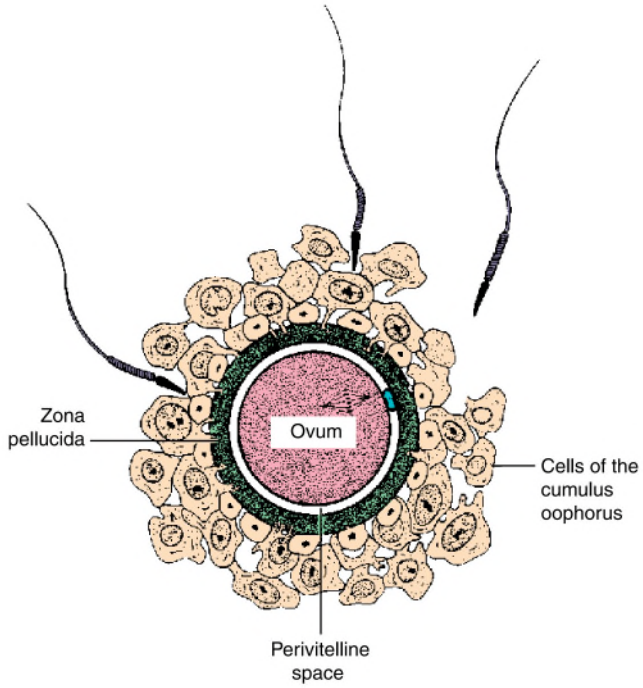


Figure 9-4 Illustration of the barriers around the recently ovulated ovum through which the capacitated sperm must pass to reach the perivitelline space and achieve activation and fertilization of the ovum.

Sperm Passage through the Zona Pellucida

The zona pellucida is an extracellular matrix composed of three glycoproteins termed ZP1, ZP2, and ZP3. Receptors on the sperm plasma membrane attach to ZP3. This ZP3 receptor binding allows the sperm to adhere to the zona pellucida and is a critical step in fertilization. It triggers the sperm head to undergo the *acrosome reaction*. An influx of calcium and a rise in pH and cAMP levels within the sperm head cause exocytosis of the acrosomal vesicle. That is, the plasma membrane of the sperm fuses with the outer acrosomal membrane, forming many small openings to the acrosome. Contents of the acrosome, which are hydrolytic enzymes, spill out and degrade the zona pellucida near the sperm head. This forms a tunnel in the zona, through which the sperm begins to move (Fig. 9-5).

Degradation of the sperm plasma membrane causes the loss of ZP3 receptors. However, now the inner acrosomal membrane is exposed, and it appears to have receptors for another zona pellucida glycoprotein called ZP2. This ZP2 binding maintains the contact between egg and sperm. The sperm tail continues to beat vigorously, helping the sperm penetrate through the zona pellucida and make contact with the plasma membrane of the egg. Once the sperm has penetrated the zona pellucida, it moves through a narrow, oblique path into the *perivitelline space* (the area between the zona pellucida and the vitelline membrane, see Fig. 9-4). Penetration of the human zona pellucida by a sperm takes less than 10 min under experimental conditions.

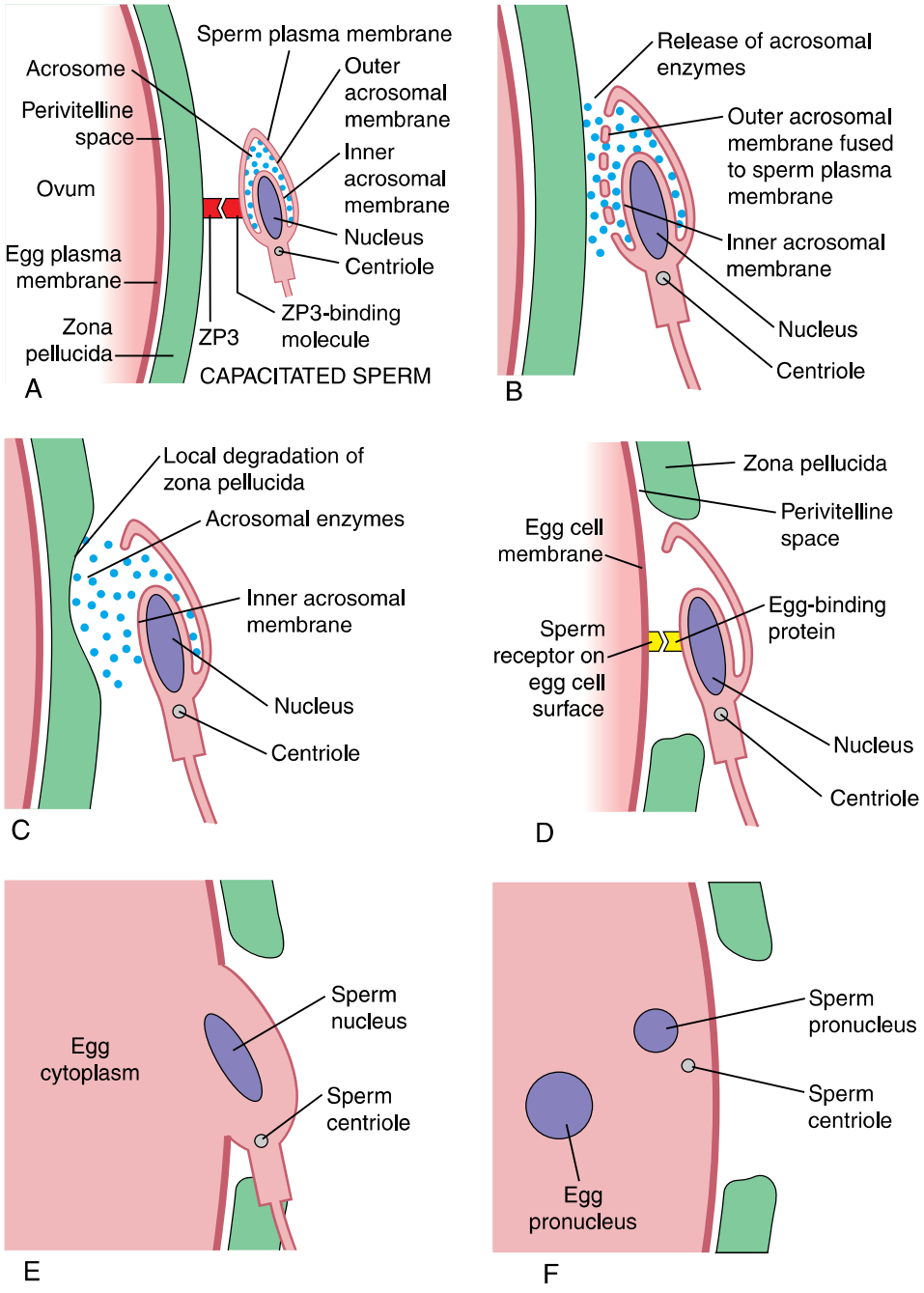


Figure 9-5 Stages of fertilization. Capacitated sperm have already passed through the cumulus oophorus surrounding the egg; for clarity, cumulus cells are not shown. (a) Proteins on the sperm plasma membrane bind to ZP3 molecules within the zona pellucida of the egg. (b) Zona binding triggers the acrosome reaction, in which the sperm plasma membrane fuses with the outer acrosomal membrane, causing exocytosis of acrosomal contents. (c) Acrosomal enzymes begin to dissolve a hole in the zona pellucida. This enzymatic degradation, accompanied by rapid sperm tail beating, moves the sperm through the zona. (d) Egg-binding proteins on the sperm cell surface bind to molecules on the egg cell membrane. (e) The sperm cell membrane fuses with the egg plasma membrane, allowing the sperm nucleus and centriole to enter the egg cytoplasm. (f) Egg and sperm pronuclei migrate toward each other in preparation for syngamy.

Sperm Attachment to the Egg Plasma Membrane

The sperm approaches the egg sideways instead of head on, and the sperm head now lies parallel to the egg cell surface within the narrow perivitelline space (Fig. 9-5). At this point, the posterior part of the sperm head attaches to the egg plasma membrane. The plasma membranes of sperm and ovum fuse, forming an opening into which the sperm nucleus, midpiece, and most of the tail sink into the egg cytoplasm. Scientists are actively investigating the molecules involved in egg–sperm adhesion and subsequent fusion. Finding the molecular basis of sperm–egg fusion may help us understand certain forms of infertility and could possibly lead to new contraceptives.

The Cortical Reaction

Once a sperm has entered the egg, it is imperative that no other sperm be permitted to fertilize it. If additional sperm were allowed to enter the egg, the extra genetic material they carry would disrupt normal development, and the resulting polyploid embryo would die. To prevent *polyspermy* (fertilization by more than one sperm), the egg now mounts a defense. Just underneath the plasma membrane of the egg lie small, membrane-bound vesicles called *cortical granules*. At fertilization, there is a sudden, dramatic burst in available free calcium in the egg cytoplasm as it is released from cytoplasmic storage. The rise in calcium causes cortical granule membranes to fuse with the adjacent cell membrane. Thus, the cortical granules open to the exterior and release their contents into the perivitelline space. Included in the cortical granule contents are enzymes that act on constituents of the zona pellucida. These enzymes alter ZP2 and ZP3, destroying their receptor sites for the sperm head. Thus, no additional sperm can attach to the zona pellucida to gain access to the egg.

The cortical reaction is the first step in a series of biochemical and physical changes in the egg known as *egg activation*. These rapid changes begin just after fertilization and are preparations for early embryonic development. In addition to the cortical reaction, egg activation involves completion of meiosis, increase in egg metabolism, synthesis of protein, RNA, and DNA, and preparation for the first mitotic division. All of these essential first steps in development are dependent on the initial rise in free calcium. We do not know exactly how fertilization initiates a calcium rise in the egg. One theory (the *receptor hypothesis*) suggests that binding of a sperm to an egg receptor induces biochemical changes in the egg cytoplasm that cause release of stored calcium. An alternative idea (the *cytoplasmic factor hypothesis*) is that as the sperm enters the egg cytoplasm, it carries a factor that causes free calcium to be released. Laboratory experiments lend support for each of these hypotheses, but the actual mechanism that occurs during normal fertilization remains unknown.

Completion of the Second Meiotic Division

The ovulated egg is arrested in the second meiotic division and still has a duplicated set of chromosomes. Before merging with sperm DNA, the egg must complete its second meiotic division and jettison one set of its chromosomes.

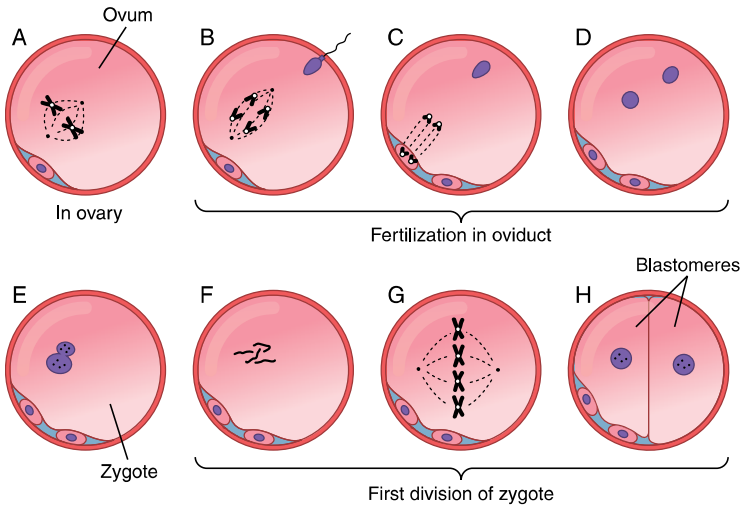


Figure 9-6 The nucleus of the ovulated egg is haploid and its chromosomes are arrested in the second meiotic division (1). The first polar body may divide into two small cells (1), one of which is pictured in further figures. Sperm penetration activates the egg so that the second meiotic division is completed (2, 3) and a second polar body is formed (4). The egg and sperm pronuclei then fuse (5) and the resultant diploid zygote now divides mitotically (6, 7) to form a two-cell embryo (8) consisting of two blastomeres. Note that only two chromosomes are shown in (1), even though there should be 23.

At fertilization, the rise in free calcium activates the egg nucleus to complete meiosis, and a second polar body is produced, removing the extra set of chromosomes from the egg. The second polar body can often be seen in the perivitelline space before it degenerates (Fig. 9-6).

Formation and Fusion of Sperm and Egg Pronuclei

Soon after the sperm nucleus enters the egg, its nuclear membrane breaks down. The sperm DNA decondenses as a result of exposure to factors in the egg cytoplasm. A new membrane then forms to enclose the *sperm pronucleus*. Sperm and egg pronuclei begin to migrate toward each other, replicating their DNA as they move. As they approach each other, their nuclear membranes break down and the two duplicated sets of chromosomes aggregate. Syngamy (merging of the two haploid genomes) has now occurred, and the fertilized egg (*zygote*) is the beginning of a new individual. In mammals, it takes about 12 h from the beginning of egg activation to pronuclear fusion. The centrosome contributed by the sperm organizes a mitotic spindle, and chromosomes now begin to line up at the metaphase plate. The zygote next divides mitotically, and two identical daughter cells, termed blastomeres, are formed (Fig. 9-6). Embryonic development has commenced.

We have seen that the sperm contributes its haploid chromosomes and centrosome to the zygote. The sperm tail disintegrates in the egg cytoplasm. What happens to the sperm mitochondria? It has long been known that the approximately 100 mitochondria brought by each sperm into an egg disappear soon after fertilization. Recent studies have demonstrated how this occurs. During spermatogenesis, sperm mitochondria are tagged with a protein called *ubiquitin*, a molecule

used by all cells to mark proteins slated for destruction. These tagged paternal mitochondria are then destroyed and recycled by the egg after fertilization. Thus, all of our mitochondria are inherited from our mothers. Maternal inheritance of DNA-containing mitochondria has been a useful way to trace human origins.

Chapter 9, Box 2: Sperm Hitchhikers

Deprived of all but a scant amount of cytoplasm during the latter stages of spermatogenesis, the human sperm has, until recently, been considered to contribute nothing to the ovum except for its nuclear DNA and centriole. However, we know that factors carried by the sperm play active roles in the fertilization process. Some men with sperm apparently normal in shape, motility, and abundance still are infertile if they lack these biochemical factors. From the text, you know that some of these factors are enzymes such as hyaluronidase and acrosomal, enzymes necessary to break through the layers of cumulus cells and the zona pellucida before reaching the egg surface. Also necessary are zona-binding proteins on the surface of the sperm cell membrane and inner acrosomal membrane. However, these are not all of the players in the process of fertilization, and some sperm "hitchhikers" may also be important for normal development of the egg and embryo.

For example, the sperm head contains a protein, *fertilin-β*, on its surface. After the sperm penetrates the zona pellucida, the tip of its head approaches the vitelline membrane. Then the head turns laterally so that one side of the sperm head attaches to the vitelline membrane (see text). Fertilin-β appears to mediate this lateral attachment. If this protein is absent, fertilization does not occur because sperm–oocyte binding is inhibited. Fertilin-deficient sperm also have a reduced ability to bind to the zona, and our understanding of the normal action of fertilin is still evolving.

When the sperm penetrates the egg, waves of stored calcium ions are released in the egg cytoplasm. This sudden increase in calcium triggers egg activation (cortical granule release and reinitiation of meiosis). Scientists have long speculated that the trigger for calcium release is carried by the sperm. Researchers have found that the sea urchin sperm head contains an enzyme that can synthesize *nitric oxide*. This gas is injected into the egg at fertilization and can set off a calcium surge. It remains to be seen if a similar mechanism operates in humans. Study of human eggs has revealed that *phospholipase C* is carried by sperm into the egg. It also can cause the waves of calcium release and egg activation.

Ribonucleic acid (RNA) is produced when cells read the DNA sequences coded by the genes and transcribe these messages. During the later stages of spermatogenesis, sperm DNA becomes tightly compressed and gene expression ceases. However, scientists have found that sperm RNA is still present in the mature sperm even at fertilization. This is especially surprising because the sperm cytoplasm is virtually gone. Sperm cells contain an amazing repertoire of RNAs. It turns out that about 3000 of the 20,000–25,000 human genes are represented by sperm RNA. Some of the mRNAs represent known genes, others are unknown, and some of the RNAs do not code for proteins. Many types of mRNAs are found in the sperm cell nucleus.

Most of these 3000 transcripts are probably leftover RNA instructions for building the sperm cell during the process of spermatogenesis. However, scientists have identified six RNAs present in the spermatozoa but not in the unfertilized egg. They then asked if these transcripts are carried into the egg at fertilization. If the sperm delivers RNAs into the egg at fertilization, one would expect to find these RNA sequences in the sperm and in the zygote, but not in the unfertilized egg. Using cDNA probes, they found two sperm RNA sequences that are delivered to the egg at fertilization.

What happens to RNAs delivered by the sperm? Possibly they are simply destroyed by

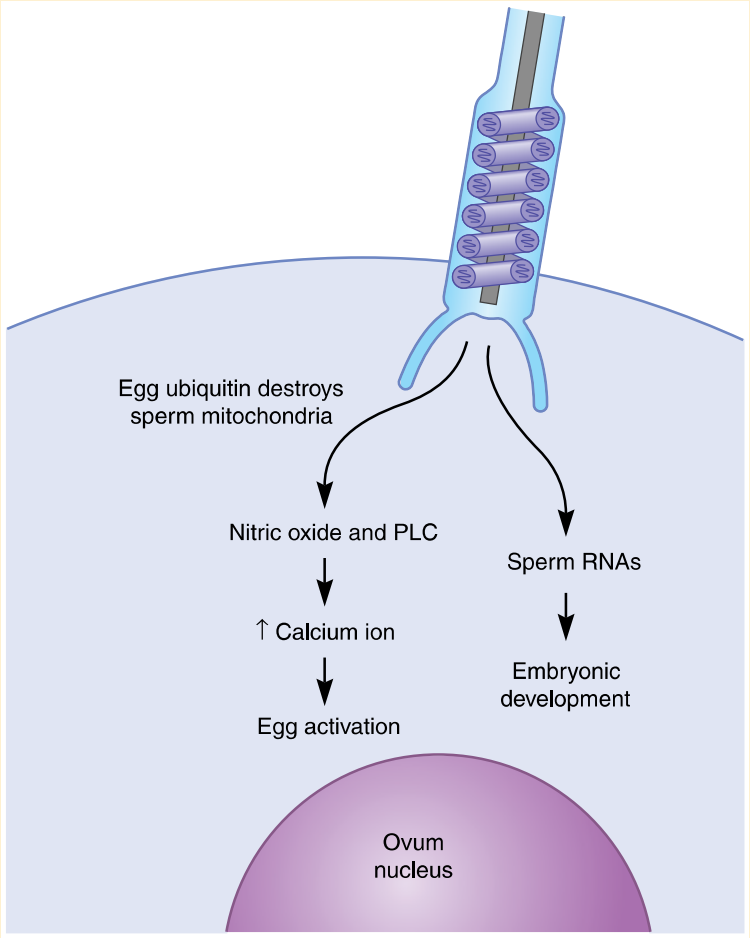
Continued on next page.

Chapter 9, Box 2 continued.

the egg cytoplasm. However, it is also possible that these RNAs act as instructions for early embryonic development and that they are needed to launch the developmental program of the zygote. In fact, one of the mRNA transcripts delivered to the egg codes for *clusterin*, which has been implicated in cell–cell interactions, membrane recycling, and regulation of apoptosis (programmed cell death), processes central to embryonic development.

Preliminary evidence shows that the sperm of some infertile men lack some of the RNAs carried by sperm of fertile men. In fact, it is thought that a treatment that lowers or eliminates the RNAs from the sperm of fertile men may render them infertile, thus providing a potential male contraceptive method. Some scientists use the absence of male RNAs as a possible explanation of why embryonic development is so poor in most cases of cloning and in all cases of human parthenogenesis; neither process involves sperm. However, others cite the occasional success of cloning to argue against an important role for sperm RNAs.

As new sperm molecules are discovered, the role of the sperm has expanded from simply delivering a haploid genome to the egg to essential roles in the fertilization process and perhaps important roles in egg activation and early embryonic development as well.



Possible influences of the sperm cell on the egg and/or early embryo in addition to the contribution of its haploid nucleus. For clarity, the sperm cell is shown oriented at right angles to the egg cytoplasm.

Chemical Inhibition of Fertilization

In the future, it may be possible to block fertilization by interfering with steps in the fertilization process. A search for vaccinations against sperm, egg, or the early embryo has long been underway. More recently, studies have focused on specific ways to thwart the actions of sperm cells, either by immobilizing them or by preventing them from undergoing the acrosome reaction, binding to the zona pellucida, or fusing with the egg cell membrane. Some of these potential future contraceptive methods are discussed in Chapter 14.

Sex Ratios

As discussed in Chapter 5, the normal chromosome number in humans is 46 (2N, diploid). Females have 22 pairs of autosomes and two X chromosomes. Males have 22 pairs of autosomes and an X and Y chromosome. The genes for male sex determination are carried on the Y chromosome. Thus, embryos without a Y chromosome are female.

As a result of meiosis in the adult testis, one diploid male germ cell (spermatogonium) gives rise to four haploid spermatozoa (see Chapter 4). Two of these spermatozoa will have 22 autosomes and a Y chromosome, whereas the other two will have 22 autosomes and an X chromosome. If a Y-bearing sperm (22Y) fertilizes an ovum (with 22 autosomes and an X chromosome), the embryo will be male; if an X-bearing sperm (22X) fertilizes an ovum, the offspring will be female. Thus, given an equal chance of X and Y sperm to fertilize, the sex ratio of embryos should be 100:100 (Fig. 9-7). However, the ratio of male to female embryos at conception (the *primary sex ratio*) is about 120:100. This ratio is based on the sexes of early aborted embryos. It is assumed that this means a greater fertilization rate by Y sperm than X sperm, perhaps because Y sperm are lighter and faster swimmers than X sperm.

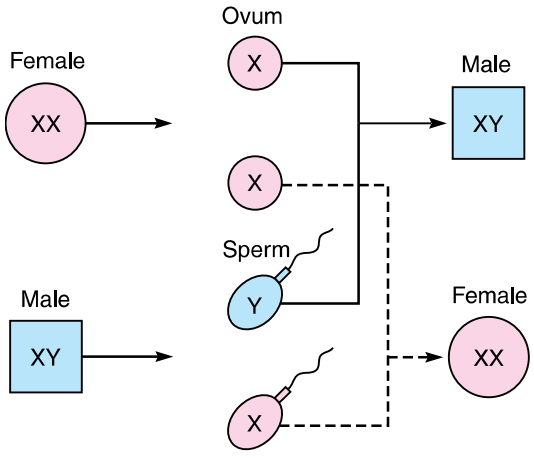


Figure 9-7 The chromosomal basis for the existence of an equal number of X and Y sperm, and thus a theoretical primary sex ratio of 100:100. As discussed in the text, this theoretical ratio is not borne out, and more embryos are male than female.

However, female embryos may die more frequently at an earlier age than male embryos or more X sperm may die in the female reproductive tract than Y sperm. The sex ratio of male births to female births (the *secondary sex ratio*) is 105:100. Thus, for reasons not yet understood, male fetuses suffer a greater mortality than female fetuses in the uterus.

Sex Preselection

Couples who desire to choose the sex of their baby may now do so. A relatively new technology (the *microsort method*) is the most effective procedure yet devised at separating X-bearing and Y-bearing sperm. It takes advantage of the fact that the large X chromosome has considerably more DNA than the tiny Y chromosome. A sperm sample is first collected from the prospective father. Then, the sperm cells are treated with a fluorescent dye that attaches to DNA and glows under laser light. Sperm with more DNA, scientists reasoned, would glow more brightly. Although X sperm have only 2.8% more DNA than those carrying a Y chromosome, the difference in brightness is sufficient to be distinguished by a light detector. The tagged sperm are sent through a very narrow tube with a diameter wide enough to allow only one sperm cell at a time. As sperm move through the tube, they are illuminated by a laser beam. An automated mechanical sperm sorter then separates the sperm, sending X sperm down one tube and Y sperm into another. The sorted sperm can then be placed in the woman's uterus (artificial fertilization) or used for *in vitro* fertilization. Approximately 91% of sperm cells in the X-bearing tube contain an X chromosome. This procedure is only about 74% effective in selecting Y-bearing sperm. Thus, the results are not foolproof, but this method does improve the chances of producing an embryo of the desired sex, especially if a couple wishes to have a girl.

A more accurate method of ensuring the sex of a baby is *preimplantation genetic diagnosis (PGD)*, which is available at a limited number of clinics. It involves *in vitro* fertilization followed by embryo selection. The mother's eggs and father's sperm are collected, and the eggs are fertilized in the laboratory. After 3 days of development, a cell is carefully removed from each embryo and chromosomes are examined. Those carrying a Y chromosome are separated from non-Y-bearing embryos. Only embryos of the desired sex are implanted into the mother's uterus. Although nearly 100% accurate, this procedure is more invasive, expensive, and controversial than sperm-sorting techniques.

Why would parents wish to preselect the sex of their offspring? One reason would be to avoid sex-linked genetic diseases, which are more likely to occur in males. Parents may also wish to balance their families or they may simply prefer to have a child of a given sex. Some have expressed concerns that the ability to select a baby's sex may be the first step to "designer children" chosen for other traits such as height, IQ, athletic, or musical ability. Others fear that widespread sex selection would lead to a gender imbalance in society and cause social problems. In fact, a preference for baby boys in China has led to a significant shift in the sex ratio in some areas of the country. In such cultures where boys are valued more highly than girls, the ability to select sex before fertilization could avoid costly and ethically controversial practices such as amniocentesis (genetic screening for sex), selective abortion, and even infanticide. In the United States, sperm selection likely would not lead to overall gender imbalance, as family preference

for a girl or a boy baby is split more evenly. Finally, the ability to preselect a child's sex may help families limit their size. For example, using gender selection technology, a family with three boys could increase their likelihood of having a girl as their fourth and last child instead of continuing to have babies until a girl was conceived. However, the present high cost of the microsort and PGD methods likely will limit the practice of sex preselection in the foreseeable future.

Multiple Embryos

Twins occur in about 1 of every 80 or 90 pregnancies. When two ova are released and each is fertilized by a different sperm, *fraternal twins* are produced. These twins are *dizygotic* (the products of two different zygotes) and can be the same or different sex. Fraternal twins, which are *nonidentical* and are as different from each other as are nontwin brothers and sisters, account for two-thirds of all twins. The incidence of dizygotic twins is influenced by race and by inherited factors from the mother (not the father). Fraternal twins are more common in older mothers.

Identical twins, which are rarer than fraternal twins, usually occur when an early embryo divides into two. These twins are *monozygotic* (derived from one zygote) and are identical genetically. The incidence of identical twins is not related to race, inheritance, or age of the mother. Rarely, identical twins are *conjoined* (i.e., they fail to separate completely during embryonic development). These are called *Siamese twins*, after the first publicized Siamese twins, "Chang" and "Eng" (1811–1874), born in Siam of Chinese extraction. They were united at the chest by a thick mass of flesh. Some Siamese twins have been separated surgically after birth. For more on twin pregnancies, see Chapter 10.

When the number of embryos is greater than two (e.g., triplets, quadruplets), all are usually of multizygotic origin; in a few cases, some are multizygotic and some are monozygotic.

Parthenogenesis

Is it possible that an embryo can develop in a human female without previous fertilization? Embryonic development from an ovum not previously stimulated or penetrated by a sperm is called *parthenogenesis*. Such "virgin birth" is common in many insects, in some fish, amphibians, and reptiles, and in a strain of domestic turkeys. In addition, parthenogenetic mouse embryos can be produced in the laboratory, but they do not develop to term. There is no proven case of a parthenogenetic birth in humans. If parthenogenesis could occur, reduction division in the oocyte must not occur, the offspring would always be female, and the child would be genetically identical to the mother.

Chromosomal Aberrations

Errors of meiosis or fertilization can produce embryos with chromosomal aberrations. More than 90% of these embryos are aborted spontaneously, usually within the first trimester. In fact, 42% of embryos or fetuses that are

aborted spontaneously have chromosomal abnormalities. A few fetuses with chromosomal defects, however, are born; about 1 out of every 100 newborns has such a defect. It must be emphasized that some of these disorders are not inherited in the strictest sense because the genes of the parents do not govern their occurrence.

In rare cases, one sperm will fertilize the ovum and a second sperm will fertilize the polar body. The two fertilized cells then form an embryo that is a genetic mosaic in that half of its cells will have a different genetic makeup from the other half. This condition also can occur when the haploid ovum divides into two cells and each cell is then fertilized by a separate sperm. If an X and a Y sperm were involved, half of the cells of an embryo would be male and half female, resulting in an intersex (see Chapter 5).

One kind of chromosomal aberration occurs when fertilization fails to activate the second meiotic division in the ovum. Thus, there is no egg pronucleus and the embryo develops with only one set of chromosomes (haploid) and genes of the male only. This process of embryonic formation is termed *androgenesis*. A similar situation occurs when the ovum pronucleus develops normally, but the sperm pronucleus does not form. In this case, called *gynogenesis*, the embryo also is haploid but has only the female's genes. Both of these conditions are lethal after only a few cell divisions in the embryo.

In contrast to the previously mentioned conditions, some embryos may develop with triploid cells (3N) that have 69 chromosomes (three complete sets). *Triploidy* can occur in at least three ways. First, sperm penetrating the ovum may be the product of a failure of reduction division during meiosis in the testis, and thus it has 46 instead of the normal 23 chromosomes. When this sperm fertilizes a haploid ovum, a triploid embryo develops. Second, even though mechanisms to prevent polyspermy are present, these mechanisms are not failsafe. Thus, two haploid sperm can penetrate a single ovum (polyspermy) and both of their pronuclei then fuse with the haploid ovum pronucleus. Finally, reduction division (meiosis) may not have occurred in the oocyte, and the resultant diploid female pronucleus then fuses with a haploid sperm pronucleus to produce a triploid zygote.

The excess dosage of genes in triploid embryos tends to be less destructive than when there are too few genes, as in androgenesis or gynogenesis. Most triploid embryos develop to about the third month of pregnancy before aborting spontaneously. The very few triploid fetuses that survive to term are malformed and are stillborn or die soon after birth. Less than 1% of all human embryos are triploid.

Another error in fertilization results in embryos with either one too many (47) or one too few (45) chromosomes in their cells; these conditions are collectively called *aneuploidy*. This happens when there is aberrant chromosome movement during the first or second meiotic division in the testis or ovary or in the first cleavage division of the zygote. That is, a pair of hromosomes fails to separate during division, with both members going to one daughter cell (*nondisjunction*). The resultant cell has 47 chromosomes, and the cell coming up short has only 45. Thus, the aneuploid condition can be either *monosomic* (45 chromosomes) or *trisomic* (47 chromosomes).

Most monosomic embryos abort spontaneously early in their development. An exception, however, is when monosomy for a sex chromosome occurs. That is, each cell has only a single sex chromosome, either an X or a Y. About 98% of

these embryos abort, but a few with one X (XO condition; Turner's syndrome) are born as sterile females with short stature and physical defects (see Chapter 5). Only 1 in 3500 living females has this syndrome.

Most trisomic embryos die in the second or third month of pregnancy and abort spontaneously; 20% of miscarried fetuses are trisomic. Some, however, are born with severe physical and mental defects. The most common trisomic condition in infants is *Down syndrome*, also called *Mongolism*, a condition in which the cells of the individual are trisomic for chromosome number 21. Children with Down syndrome exhibit abnormal body development and severe mental retardation.

For some as yet unknown reasons, the gametes of older men and women are more likely to produce trisomic embryos. The chances are 1 in 1000 for having a trisomic embryo for women under 35, but are 1 in 200 for 35-year-old women and 1 in 15 for 45-year-old women. Women over 35 have 15% of all babies but 50% of all Down's syndrome children. Therefore, it is recommended that women in their midthirties consider having the cells of their fetus examined by amniocentesis or chorionic villus biopsy (see Chapter 10) for evidence of chromosomal abnormalities. If certain chromosomal aberrations are found, induced abortion might be considered (see Chapter 15). It used to be thought that errors in meiosis in oocytes of older women were the main cause of trisomy. Recently, however, we have become aware that about one-fifth of trisomic infants are caused by chromosomal abnormalities in the sperm of older men.

As discussed in Chapter 5, nondisjunction of sex chromosomes can produce males with trisomic cells of an XXY or XYY makeup. In the former condition, Klinefelter's syndrome, males are sterile and have female-like breasts. About 1 out of 600 males is born with this condition. In the latter "supermale" condition (XYY), males are very tall and often have acne. These males tend to exhibit mental and social adjustment problems at a higher percentage than normal XY males. One in 2000 males has XYY cells. Some statistical evidence exists that the percentage of XYY males (1.8 to 12.0%) in penal institutions is greater than their percentage (0.14 to 0.38%) in the general population. Some controversy, however, surrounds these studies and it is not clear if the greater maladaptive behavior of XYY males is a direct result of their chromosomal abnormality or is due to social problems they had when growing up because of their unusual physical appearance. Apparently the elevated crime rate of XYY men is not related to aggression but may be related to low intelligence. Women with nondisjunction of the X chromosome have cells that are XXX. These women are female but sterile. Cases in which males have several X chromosomes (XXXY) are due to penetration of the ovum by more than one sperm.

Sometimes a gamete contains a chromosome with an extra piece from another chromosome attached to it; this is the result of *chromosomal translocation*. The chromosome from which the piece was taken thus suffers from *chromosomal deletion*. An example of a disorder resulting from chromosomal deletion is the *cri du chat* (French for "cry of the cat") syndrome, in which a piece of chromosome 5 is missing. These children are born with a small head, widely separated eyes, low-set ears, and mental retardation. When they cry, it sounds like a hungry kitten. Human kidney cancer has also been linked to an inherited chromosomal translocation in which a piece of chromosome 3 is hooked onto chromosome 8.

An inherited disorder of the X chromosome (*fragile X syndrome*) is the second leading cause of mental retardation. In these people, the X chromosome (in either sex) has an abnormally long, fragile arm. In this disorder, mental retardation is less severe in females than in males.

Chapter Summary

After sperm mature in the epididymides, they move down the vasa deferentia. Seminal plasma consists of secretions from male sex accessory glands. These secretions are added to the sperm to form semen (seminal fluid), which leaves the male urethra during ejaculation. Seminal plasma contains substances necessary for sperm movement, maturation, and maintenance.

About 66 million sperm are present in each milliliter of semen. Some of these sperm are abnormal and die. A healthy sperm is made up of a head (nucleus plus acrosome), neck, midpiece, and tail. After insemination of the female, the sperm move through the vagina, cervix, uterus, and into the oviduct. While in the uterus and oviduct, sperm acquire the ability to fertilize (capacitation) and are activated so that their tails beat more rapidly. Meanwhile, the ovulated ovum moves down the oviduct, and the sperm and ovum meet at the ampullary–isthmic junction of the oviduct, where fertilization occurs.

Before penetrating the ovum, a sperm moves first through the cumulus oophorus and zona pellucida. As it binds to the ZP3 glycoprotein on the zona pellucida, it undergoes the acrosome reaction, during which the sperm acrosome releases enzymes that help dissolve the zona. Once the sperm enters the ovum, it causes the completion of oocyte meiosis and the cortical reaction, which produces changes in the zona pellucida that act as a barrier to polyspermy. The haploid sperm pronucleus and egg pronucleus then merge, and a zygote is formed. In the future, certain chemicals may be used to block fertilization as a method of birth control.

Chromosomal sex is determined at fertilization, and couples may now be able to choose their baby's sex. Identical twins (monozygotic twins) are formed when a single sperm fertilizes a single ovum, after which the embryo divides into two. Fraternal twins (dizygotic twins) are formed by the fertilization of two separate eggs and sperm. Although several nonmammalian animal species can have offspring without fertilization (parthenogenesis), this has not occurred in humans.

Chromosomal errors that occur before or during fertilization can result in formation of an embryo that is haploid, triploid, aneuploid, or containing one or more chromosomes with added or deleted genetic material. Most embryos with serious chromosomal errors die early in development, but some genetic errors cause mild to severe disorders in humans.

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